

01

Signal transduction

01-01/P

NUCLEAR ASSOCIATION OF IRF5 AND IRAK-1

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The transcription factor IRF5 is involved in signaling emanating from Toll-like Receptors. Whether the expression of inflammatory cytokines or type I interferons are regulated by the TLR – IRF5 pathway is discussed controversially. Consistently is the finding that IRF5 is involved in the MyD88 dependent signaling pathway: it coimmunoprecipitates with MyD88 and TRAF6, both members of the initial signal transduction complex of the Toll/Interleukin-1 Receptor family, and functionally acts downstream of this complex. Centrally involved in formation of the initial signal transduction complex is the protein-kinase IRAK-1. IRAK-1 relays the signal generated at the receptor towards TRAF6 by interacting with the upstream partner MyD88 and TRAF6 itself, its downstream adapter, via its N-terminal death domain and its C-terminal region, respectively. In the present study we demonstrate that the transcription factor IRF5 not only interacts with MyD88 or TRAF6, but also with IRAK-1. In coimmunoprecipitation experiments we are able to show that IRF5 interacts with the kinase domain of IRAK-1 and that this association is inhibited by phosphorylation of IRAK-1 within the ProST domain. We next asked whether the association between IRF5 and IRAK-1 might also occur in the nucleus, the site of action of the transcription factor IRF5. By transient expression of IRF5 together with kinase inactive IRAK-1 and fractionation of cellular lysates we are able to show that the main proportion of IRF5 remains within the cytosol, however, IRF5 coimmunoprecipitating with kinase inactive IRAK-1 is found exclusively in the nuclear fraction. Functional consequences of the observed interaction between IRF5 and IRAK-1 are currently under investigation by employing an IRAK-1-deficient cell line.

P: poster

O: oral presentation

01-02/O

THE CRITICAL ROLE OF GP130-DEPENDENT STAT3 HYPERACTIVATION IN TUMOUR GENESIS AND INFLAMMATION

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The latent transcription factor STAT3 has been shown to be critical for the regulation of cell survival, migration and proliferation, and aberrant or persistent STAT3 activation is a common hallmark of many hyper-proliferative disorders, including solid and liquid tumours. STAT3 transduces signals from many growth factor and cytokine receptors, and in particular plays a predominant role in transmitting signals via gp130, the common signalling receptor subunit for the interleukin (IL)-6 cytokine family. We have previously generated gp130^{Y757E/Y757F} mice homozygous for a knock-in mutation at tyrosine 757 in gp130 that abolishes a negative feedback mechanism to terminate signalling from the receptor, which in turn leads to exaggerated gp130-dependent activation of STAT1 and STAT3 [1]. As a consequence, these mice have a shortened life-span and develop a complex phenotype with 100% penetrance from 6 weeks of age onwards, highlighted by multi-organ inflammation, splenomegaly, lymphadenopathy and gastric adenomatous hyperplasia [1, 2]. To determine whether exaggerated STAT3 activation was responsible for the phenotype in gp130^{Y757E/Y757F} mice, we generated gp130^{Y757E/Y757F} mice on a STAT3 heterozygous background (gp130^{Y757E/Y757F}; STAT3^{He}) to genetically reduce the level of gp130-dependent STAT3 activation, but not STAT1 activation. Indeed, compound gp130^{Y757E/Y757F}; STAT3^{He} mice live longer, and display a normal hematopoietic compartment, normalised inflammatory responses, and a striking reduction in the formation of gastric lesions, suggesting a broad physiological role for gp130-controlled tissue homeostasis that depends on the expression level of the underlying STAT3 response genes [3]. Finally, we reveal how exaggerated gp130-dependent STAT3 activation in gp130^{Y757E/Y757F} mice highlights cross-talk between common signalling pathways utilised by heterologous receptor systems, such as TGFβ, with particular relevance to gastro-intestinal homeostasis.

References

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01-03/O

SUPPRESSOR OF CYTOKINE SIGNALLING (SOCS)-1 NEGATIVELY REGULATES TOLL-LIKE RECEPTOR SIGNALLING BY MEDIATING MAL DEGRADATION**A Mansell¹, R Smith¹, SL Doyle², P Gray², JE Fenner¹, PJ Crack¹, SE Nicholson³, DJ Hilton³, LAJ O'Neill², PJ Hertzog¹**¹Centre for Functional Genomics and Human Disease, Monash Institute of Medical Research, Monash University, Melbourne, Victoria, Australia; ²School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland; ³Walter and Eliza Hall Institute, Parkville, Victoria, Australia

The ability to recognize and respond to pathogen threat is a fundamental requirement of the host to ensure survival. In humans, the innate immune system provides the initial response to this threat via the Toll-like receptor (TLR) family of pattern recognition receptors.

TLR activation however is a double edged sword. It is essential for provoking the innate immune response and enhancing the adaptive immunity against pathogens, but members of the TLR family are also involved in the pathogenesis of autoimmune, chronic inflammatory and infectious diseases. One of the most debilitating diseases is severe sepsis or septic shock induced by LPS. TLR activation has also been implicated in rheumatoid arthritis, chronic obstructive pulmonary disease, diabetes, asthma, autoimmune encephalomyelitis, cardiomyopathy, lupus and atherosclerosis. Therefore, the intensity and duration of TLR responses must be tightly controlled.

The adaptor protein Mal is specifically involved in signalling via TLRs 2 and 4. We have recently characterized a mechanism of degradation of Mal by SOCS-1 that occurs upon signalling by TLRs 2 and 4. Mal was found to associate with SOCS-1, which acts as an E3 ligase to mediate polyubiquitination of Mal on two N-terminal lysines; the polyubiquitination of Mal results in its degradation via the 26S proteasome. We also found that Mal undergoes tyrosine phosphorylation via activity of Brutons tyrosine kinase (Btk), which is consistent with the requirement for tyrosine phosphorylation for targets of SOCS-1 activity. Perturbed regulation of these events results in potentiated Mal-dependent NF-kappaB transactivation and prolonged pro-inflammatory response. Targeted degradation of Mal by SOCS-1 therefore regulates TLR activation of NF-kappaB, causing instead rapid (and temporary) refractoriness to prolonged TLR2/4 signalling and chronic inflammatory responses. These data identify a target of SOCS-1 that regulates TLR signalling via a mechanism distinct from an autocrine cytokine response.

01-04/P

PROFILES OF MYCOBACTERIUM AVIUM COMPLEX-INDUCED IMMUNOSUPPRESSIVE MACROPHAGES MEDIATED SUPPRESSION OF T CELL AND B CELL MITOGENIC RESPONSES**T Shimizu T¹, SS Cai¹, H Tomioka¹**¹Shimane University School of Medicine, Izumo, Japan

Refractory mycobacterial infections, especially multidrug resistant tuberculosis and *Mycobacterium avium* complex (MAC) infections, are frequently encountered in AIDS patients. Notably, in the advanced stages of mycobacterial infections, the generation of immunosuppressive macrophages (MΦs) is generally observed. These MΦ populations suppress T cell functions thereby resulting in the severe suppression of cellular immunity in the advanced stages of mycobacterioses. In this context, we previously found that MAC-induced immunosuppressive macrophages (MAC-MΦs) exhibit suppressor activity against concanavalin A-induced T cell mitogenesis (T cell Con A mitogenesis) by producing reactive nitrogen intermediates (RNIs) and other suppressor mediators as well as through direct cell contact with target cells. In this study, we examined the profiles of MAC-MΦ-mediated suppression of lipopolysaccharide-induced B cell mitogenesis (B cell LPS mitogenesis) and found the following: 1) While N^G-monomethyl-L-arginine and carboxy-PTIO effectively blocked the MAC-MΦ's suppressor activity against T cell Con A mitogenesis, the activity of MAC-MΦs against B cell LPS mitogenesis was only weakly affected

by these NO-reducing agents. 2) B cell LPS mitogenesis was much more susceptible to MAC-MΦ-derived reactive oxygen intermediates than T cell Con A mitogenesis. 3) B cell LPS mitogenesis was less susceptible to the inhibitory effects of other MAC-MΦ-derived suppressor mediators, including free fatty acids, TGF-β and prostaglandin E₂, than T cell Con A mitogenesis. 4) MAC-MΦ's suppressor activity was strongly dependent on B7-1 like molecule-mediated cell contact with target cells only in the case of T cell Con A mitogenesis. These findings suggest that the mechanisms for MAC-MΦ-mediated suppression of B cell mitogenesis differ from those for MAC-MΦ-mediated suppression of T cell mitogenesis, in terms of the roles of the suppressor mediators and the profiles of suppressor signal transmission to target cells.

01-05/P

PHYSICAL AND FUNCTIONAL ASSOCIATIONS BETWEEN 3-PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE-1 (PDK1) AND SMAD PROTEINS**Hyun-A Seong, Haiyoung Jung, Hyunjung Ha***Department of Biochemistry, School of Life Sciences, Research Center for Bioresource and Health, Biotechnology Research Institute, Chungbuk National University, Cheongju 361-763, Republic of Korea*

3-phosphoinositide-dependent protein kinase-1 (PDK1) is a serine/threonine kinase that plays a central role in activating AGC subfamily of protein kinases. In the present study we show that PDK1 coimmunoprecipitates Smad proteins, including Smad2, Smad3, Smad4, and Smad7, and that this association is mediated by the pleckstrin homology (PH) domain of PDK1, not by the catalytic kinase domain. We also demonstrated that Smad proteins, which are binding partners of PDK1, can be directly phosphorylated by PDK1. The complex formation between PDK1 and Smad proteins was induced by insulin treatment, but inhibited by TGF-β treatment, suggesting a functional link between PDK1 and TGF-β signaling pathways. Activity analysis of the interacting proteins showed that Smad proteins enhanced PDK1 kinase activity through the removal of 14-3-3, a negative regulator of PDK1, from PDK1-14-3-3 complex. Consistently, knockdown of the endogenous Smad proteins, which include Smad3 and Smad7, by the transfection of the small-interfering RNA (siRNA) showed an opposite result in the PDK1 activity, PKB/Akt Phosphorylation, and Bad phosphorylation. Moreover, the coexpression of PDK1 showed an inhibition of TGF-β-mediated transcription in a dose-dependent manner. In addition, confocal microscopic studies showed that the coexpression of PDK1 prevented the nuclear translocation of Smad3 and Smad4, and the redistribution of Smad7 from nucleus to cytoplasm in response to TGF-β. Taken together, our results suggest that Smad proteins can be potential intermediates linking between PI3K/PDK1- and TGF-β-mediated signal pathways.

01-06/O

RETINOIC ACID INDUCIBLE GENE I (RIG-I) PATHWAY MEDIATES INFLUENZA A VIRUS-INDUCED EXPRESSION OF ANTIVIRAL CYTOKINE GENES**Matikainen S¹, Sirén J¹, Imaizumi T², Krug RM³, Lin R⁴, Hiscott J⁴, Julkunen I¹**¹Department of Viral Diseases and Immunology, National Public Health Institute, Helsinki, Finland; ²Department of Vascular Biology, Hirosaki University School of Medicine, Hirosaki, Japan; ³Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, USA; ⁴Departments of Microbiology & Immunology, Medicine and Oncology, McGill University, Montreal, Canada

Epithelial cells of the lung are the primary targets for respiratory viruses. Virus-encoded ssRNA can activate Toll-like receptors (TLRs) 7 and 8 whereas dsRNA is bound by TLR3 and RNA helicases, retinoic acid inducible gene-I (RIG-I) and mda-5. This recognition leads to the activation of host cell cytokine gene expression. Here we have studied the regulation of influenza A and Sendai virus-induced IFN-α, IFN-β,

IL-28, and IL-29 gene expression in human lung A549 epithelial cells. Sendai virus infection readily activated the expression of IFN- α , IFN- β , IL-28, IL-29 genes, whereas influenza A virus-induced activation of these genes was mainly dependent on pretreatment of cells with IFN- α or TNF- α . IFN- α and TNF- α induced the expression of RIG-I, TLR3, MyD88, TRIF and IRF7 genes. Ectopic expression of constitutively active form of RIG-I (Δ RIG-I), MAVS/Cardif or IKKe, but not that of TLR3, enhanced the expression IFN- β , IL-28, and IL-29 genes or induced IFN- β promoter activity. Transfection of HEK293 cells with RIG-I expression plasmid, but not that of TLR3, mediated influenza A virus-induced IFN- β gene expression indicating that the RIG-I signaling pathway is activated by influenza A virus. Furthermore, dominant negative form of RIG-I inhibited influenza A virus-induced IFN- β promoter activity in IFN- α and TNF- α -pretreated cells. In conclusion, IFN- α and TNF- α enhanced the expression of the components of TLR and RIG-I signalling pathways but RIG-I was identified as the central regulator of influenza A virus-induced expression of antiviral cytokines in human lung epithelial cells.

01-07/O

STAT3 NUCLEAR LOCALIZATION INDEPENDENT OF TYROSINE PHOSPHORYLATION AND SUBSTRATE RECOGNITION BY BREAST TUMOR KINASE

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The STAT3 transcription factor is a member of the family of Signal Transducers and Activators of Transcription that function as DNA-binding factors to regulate gene expression. STAT3 can act as an oncogene, and its function has been shown to be critical for cellular transformation by a number of oncogenic tyrosine kinases. The role of STAT3 as a DNA-binding transcription factor depends on its ability to gain entrance to the nucleus. We provide evidence that STAT3 dynamically shuttles between cytoplasmic and nuclear compartments, but maintains prominent nuclear presence. Although tyrosine phosphorylation is required for STAT3 to bind to specific DNA target sites, nuclear import takes place constitutively and independently of tyrosine phosphorylation. We identify a region within the coiled-coil domain of the STAT3 molecule that is necessary for nuclear import, and demonstrate that this region is critical for its recognition by a specific import carrier importin- α 3. The nuclear presence of unphosphorylated STAT3 suggested that it might be a target of a nuclear kinase. The Breast Tumor Kinase (Brk) is a non-receptor tyrosine kinase distantly related to the Src family kinase but with prominent nuclear presence. It is expressed in more than 60% of breast tumors, but the biological role of this kinase remains to be determined. We provide evidence that STAT3 is a physiological target of Brk. Results demonstrate that STAT3 is tyrosine phosphorylated and transcriptionally activated in cells expressing endogenous Brk. Other STAT members are not responsive to Brk expression. In addition, we have determined that the suppressor of cytokine signaling, SOCS3, is a negative regulator of STAT3 phosphorylation by Brk.

01-08/P

NF- κ B IS REQUIRED FOR STAT-4 EXPRESSION DURING DENDRITIC CELL MATURATION

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Although the significance of dendritic cells (DC) as regulators of innate and adaptive immunity is beyond doubt, little is known about intracellular mechanisms regulating DC functions. Microarray analysis showed that DC maturation is associated with the expression of a complex stimulus-specific gene profile involving several transcription factors. Interestingly, many inducers of DC maturation are also strong activators of NF- κ B transcription factors, suggesting that these latter regulate the expression of a specific transcriptome leading to DC maturation.

Moreover, several other transcription factors, such as AP-1 and IRFs, may play a role in the regulation of genes associated with DC maturation. Few data are available on the function of STAT factors in maturing DC. Among the STAT factors we focused on STAT-4 which is activated by two key immunoregulatory cytokines, IL-12 and IFN- β , released from maturing DC. Initially thought to be restricted to the lymphoid lineage, STAT-4 was subsequently shown to be expressed in the myeloid compartment, mainly in activated monocytes, macrophages and DC. Here we have studied STAT-4 in human monocyte-derived DC and we demonstrated that its expression can be induced by multiple stimuli, such as the ligands for TLR-4, TLR-2 and TLR-3, different pathogens, CD40L and the pro-inflammatory cytokines tumor necrosis factor- α and interleukin-1 β . Interestingly, we found that STAT-4 is tyrosine phosphorylated in response to type I IFN, but not IL-12 in human mature DC. Cloning and functional analysis of the STAT-4 promoter showed that a NF- κ B binding site is involved in the regulation of this gene in primary human DC. The mutation of this kB site or the overexpression of the repressor I κ B α exerted an inhibitory effect on a STAT-4 promoter-driven reporter. All together these results indicate that STAT-4 can be finely tuned along DC maturation through NF- κ B activation.

01-09/P

TUMOR NECROSIS FACTOR RECEPTOR ASSOCIATED FACTOR-6 INHIBITED ACTINOMYCIN D PLUS TNF- α -INDUCED CELL DEATH

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Tumor necrosis factor (TNF) receptor associated factor (TRAF)-6 transduces signals from members of TNFR superfamily and the Toll-like receptor /interleukin-1 receptor family. While TNF- α exerts a variety of biological effects including production of inflammatory cytokines, proliferation, differentiation and cell death, the role of TRAF6 in the TNF- α -induced cell death remains unclear. Here, we show that murine embryonic fibroblasts (MEF) derived from TRAF6 knockout mice have increased sensitivity to actinomycin D (ActD) plus TNF- α -induced cell death compared with wild-type MEF. While ActD plus TNF- α -induced phosphorylation of JNK and ERK, and degradation of I κ B α were not different between TRAF6^{-/-} and wild-type MEF, slightly higher caspase-3 activity was observed in TRAF6^{-/-} MEF. In addition, reactive oxygen species (ROS) were more accumulated in TRAF6^{-/-} MEF than in wild-type MEF. To evaluate the contribution of caspase activation and/or ROS accumulation to ActD plus TNF- α -induced cell death, cells were treated with a pancaspase inhibitor, z-VAD-fmk or an antioxidant, butylated hydroxyanisole (BHA), prior to ActD plus TNF- α treatment. BHA but not z-VAD-fmk dramatically inhibited ActD plus TNF- α -induced cell death in TRAF6^{-/-} MEF. These results indicated that ActD plus TNF- α -induced cell death in TRAF6^{-/-} MEF was caspase-independent but ROS-dependent cell death. To address the contribution of apoptosis or necrosis to ActD plus TNF- α -induced cell death, cells were stained with Annexin V-FITC and propidium iodide analyzed by flow cytometry. ActD plus TNF- α -induced cell death in TRAF6^{-/-} MEF was mainly necrotic cell death. In conclusion, we assumed that ActD plus TNF- α -induced necrotic cell death in MEFs is regulated by TRAF6.

01-10/O

REQUIREMENT OF SRC FOR DOUBLE-STRANDED RNA-INDUCED SIGNALING BY TLR3: TWO STEP ACTIVATION OF NF κ B

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TLR3-mediated signaling by dsRNA leads to the activation of two major transcription factors, IRF-3 and NF- κ B. We previously reported that tyrosine phosphorylation of TLR3 is required for TLR3

to IRF-3 signaling. Here we report that the cellular tyrosine kinase, Src, is required for this process. Pharmacological inhibitors of Src or ablation of the protein by siRNA inhibited ISG56 induction in human cells. Similarly, in Wt mouse fibroblasts, but not in Src *-/-* fibroblasts, expression of TLR3 allowed dsRNA-mediated ISG54 induction. Restoration of Src in Src *-/-* fibroblasts restored TLR3 signaling. The characteristics of activation of the NF- κ B pathway were similar to those of the IRF-3 pathway: phosphorylation of only two out of five tyrosines in the cytoplasmic domain of TLR3, Tyr759 and Tyr858, was sufficient for NF- κ B activation. In dsRNA treated cells expressing the Y759F mutant of TLR3, the NF- κ B-driven genes were not induced; however, the NF- κ B pathway was partially activated. NF- κ B was released from I κ B as revealed by EMSA and it translocated to the nucleus. In spite of being in the nucleus, the NF- κ B p65 protein could not bind tightly to the promoter κ B element of the A20 gene as revealed by Chromatin-Immunoprecipitation assays. Two-dimensional gel electrophoresis demonstrated that this defect was due to incomplete phosphorylation of the NF- κ B p65 protein in cells expressing the mutant TLR3. The above observations indicate that like IRF-3 activation, NF- κ B activation is a two-step process: in the first step it is released from I κ B upon phosphorylation of the latter protein and in the second step p65 itself gets phosphorylated at multiple sites to attain its full transcriptional activity. The two steps of activation, in turn, are initiated by dsRNA-induced phosphorylation of the two tyrosine residues, 858 and 759, of TLR3.

01-11/P

MULTIPLE SIGNALING PATWAYS INVOLVED IN MYELOID AND ERYTHROID PROGENITOR CELL PROLIFERATION AND DIFFERENTIATION

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A number of studies on intracellular signaling clearly demonstrated that hematopoiesis is the cumulative result of intricately regulated signal transduction cascades that are mediated by cytokines and their cognate receptors. However, the pleiotropic nature of both cytokines and the signaling pathways they activate has prompted questions about the signal specificities that lead to the unique biological events of the particular cytokine, as well as about the lineage-specific responses elicited. In this study we investigated the signal transduction pathways associated with the clonal development of hematopoietic progenitor cells. The contribution of particular signaling molecules of protein tyrosine kinases (PTKs), MAP kinase and PI-3 kinase signaling to the growth of murine bone marrow granulocyte-macrophage (CFU-GM) and erythroid (BFU-E and CFU-E) progenitors was examined in studies performed in the presence or absence of specific signal transduction inhibitors. For CFU-GM and BFU-E progenitor assays the same cytokine combination was used expected to discriminate between cell lineage specifications, while separate clonogenic assays for early-BFU-E, and late-CFU-E erythroid progenitors enabled to discriminate the erythroid colony growth dependent on progenitors' stage of differentiation. Lineage-specific differences were obtained when inhibitors specific for receptor- or non-receptor-PTKs, as well as for the main groups of distinctly regulated MAPK cascades were used, pointing to the erythrocyte lineage as more sensitive target for the tested compounds. Differences dependent on progenitors' maturity were observed when the inhibitor of MEK/ERK transduction was used. At the same time, PI-3 kinase was required for the maintenance of both myeloid and erythroid progenitor cells function. The data obtained indicated that committed hematopoietic progenitors express certain level of constitutive signaling activity that participates in the regulation of steady-state hematopoiesis and imply to the importance of evaluating the impact of signal transduction inhibitors on normal bone marrow when used as potential therapeutic agents.

01-12/P

INVOLVEMENT OF MAPK PATHWAYS IN IL-17 INDUCED INHIBITORY EFFECT ON BONE MARROW CFU-E PROGENITORS

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Interleukin-17 (IL-17) is a T cell cytokine implicated in the regulation of hematopoiesis and inflammation. Numerous studies showed that IL-17 stimulates hematopoiesis, specifically granulopoiesis inducing expansion of committed and immature progenitors in bone marrow. Our previous results also pointed to its role in erythropoiesis, demonstrating significant stimulation of early erythroid progenitors, BFU-E and suppression of late stage erythroid progenitors, CFU-E in the bone marrow from normal mice. The mechanisms and signaling cascades whose activations are required for such effects remain unknown. In the present study we investigated whether mitogen-activated protein kinases (MAPK) pathways are engaged in IL-17 signaling and whether their function is required for the generation of the suppressive effect of IL-17 on late stage murine erythroid bone marrow progenitors. The contribution of p38, JNK and MEK1/2-ERK1/2 MAPK pathways to the growth of murine bone marrow erythroid progenitors was examined in studies performed in the presence or absence of specific signal transduction inhibitors. To define the functional role of MAPK pathways in IL-17 signaling, the effects of pharmacological inhibition of MAPKs on the induction of IL-17 responses were determined. The results demonstrated that the activation of p38 and JNK MAPK, but not ERK, was essential for the erythropoietin-stimulated CFU-E colony formation, since the p38- and JNK-specific inhibitors, SB203580 and SP600125 respectively, exhibited profound inhibitory effects on the CFU-E growth, while the inhibitor of MEK1/2-ERK1/2 signaling, PD98059, had no effect on the growth of CFU-E progenitor cells. However, when combined with IL-17, both PD98059 and SB203580 reversed the CFU-E suppression by IL-17 in dose-dependent manner, while SP600125 had no effects on IL-17-dependent hematopoietic suppression. The data obtained indicated that the inhibitory effect of IL-17 on CFU-E colony formation may result from alterations in the erythropoietin-stimulated signal transduction pathways.

01-13/P

IN VITRO TREATMENT OF HUMAN MONOCYTE-DERIVED MACROPHAGES WITH THE HIV-1 REGULATORY PROTEIN NEF ACTIVATES CELLULAR SIGNALING PATHWAYS LEADING TO TYPE I IFN PRODUCTION

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The viral protein Nef is a virulence factor that plays multiple roles during the HIV replication cycle. Nef expression induces downregulation of CD4 and MHC-I, alteration of T cell receptor signaling, pro-apoptotic effects in uninfected bystander cells and anti-apoptotic effects in infected cells. Recently it has also been shown that exogenous Nef has several effects on different cell lineages ranging from induction of apoptosis in uninfected T cells, maturation in dendritic cells and suppression of CD40-dependent immunoglobulin class switching in B cells. We have previously observed that recombinant Nef treatment of monocyte-derived macrophages (MDM) purified from healthy donors induces a pro-inflammatory state in those cells, characterized by the activation of NF- κ B and MAPKs pathways and the release of a set of chemo- and cytokines (Federico *et al.*, Blood 2001, 98:2752; Olivetta *et al.*, J. Immunology 2003, 170:1716; Percario *et al.*, J. Leuk. Biology 2003, 74:21; Fiorucci *et al.*, submitted; Mangino *et al.*, in preparation). Array and RT-PCR analyses performed on treated MDM showed that Nef is able to up-regulate the expression of IFN β mRNA and that this activation required an intact myristoylation site of the viral protein. The induction of mRNA is correlated to the phosphorylation of IRF-3 and is followed by the synthesis and the release of IFN β leading to the activation of STAT2. IFN β production as well as STAT2 and IRF-3 phosphorylation are dependent on the myristoylation of Nef. Studies using different myr⁺-recNef mutants are ongoing to identify domains involved in those events.

01-14/P**THE ROLE OF INOSITOL HEXAKISPHOSPHATE KINASE 2 IN TNF- α SIGNAL TRANSDUCTION****Morrison B¹, Bauer J¹, Tang Z¹, Lupica J², Lindner D^{1,2}***¹Center for Hematology and Oncology Molecular Therapeutics, Taussig Cancer Center; ²Department of Cancer Biology, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA*

Our laboratory was the first to report that the Inositol Hexakisphosphate Kinase 2 (IHPK2) gene played a role in apoptosis. Over expression of IHPK2 sensitizes ovarian carcinoma cell lines to the growth suppressive and apoptotic effects of IFN-beta, IFN-alpha2 treatment and gamma-irradiation. Expression of a kinase-dead mutant abrogated ~50% of growth inhibition and apoptosis induced by IFN-beta. Nuclear translocation of IHPK2 contributes to the induction of apoptosis by IFN-beta or gamma-irradiation. Our recent data suggests that IHPK2 functions to enhance cell death by inhibiting the NF- κ B survival pathway, specifically by binding and inhibiting the activity of the adapter protein TNF receptor associated factor 2 (TRAF2). The TRAF family of proteins binds to proteins clustered at the cytoplasmic terminus of death receptors, such as the TNF and Apo2L/TRAIL receptors. TRAF2 couples the death receptors that ultimately result in NF- κ B activation. IHPK2 contains four SXXE motifs, putative binding sites for TRAF2. We hypothesized that TRAF2 binding, mediated by the SXXE domains of IHPK2, would disrupt NF- κ B survival signaling. To determine whether disruption of TRAF2 binding was important in abrogating apoptosis, the four SXXE motifs of IHPK2 were point mutated singly and in combination. NIH-OVCAR-3 cells were stably co-transfected with IHPK2 mutants and TRAF2. Cell lysates were immunoprecipitated with IHPK2 mAb. Western Blot analysis demonstrated that the SXXE mutations (S347A and S359A) in combination (but not singly) dramatically reduced TRAF2 binding activity. Cells expressing these mutants were 2-6 fold more effective activating NF- κ B (measured by EMSA) compared to cells co-transfected with wild type IHPK2 and TRAF2. IHPK2 mutants also expressed increased levels of XIAP. Antiproliferative assays revealed that overexpression of IHPK2 sensitized cells to IFN-beta. In contrast, only the S347A, S359A mutants conferred resistance to IFN-beta. Therefore, IHPK2 promotes apoptosis not only via its kinase activity, but also by interfering with TRAF2 function.

01-15/O**ACT1 FUNCTIONS AS A NOVEL E3 UBIQUITIN LIGASE AND ITS DEFICIENCY LEADS TO SJOGREN'S DISEASE AND LUPUS NEPHRITIS****Youcun Qian¹, Jianhua Xiao¹, Martin Scott^{2*}, Zhijian James Chen³, Xiaoxia Li¹***¹Department of Immunology, Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44915; ²Biogen, Cambridge, MA 02142; ³Department of molecular biology, University of Texas Southwestern Medical Center at Dallas*

We previously showed that adaptor molecule Act1 functions as a negative regulator in CD40- and BAFF-mediated B cell survival. Here we report that the Act1^{-/-} mice developed secondary Sjogren's disease in association with systematic lupus erythematosus as they aged. The Act1^{-/-} mice developed signs of keratoconjunctivitis sicca (dry eye) and xerostomia (dry mouth) with lymphocyte infiltration in exocrine glands and production of anti-Ro/La autoantibodies. These mice also spontaneously developed lupus nephritis with immunoglobulin deposition in the kidney. Act1^{-/-}CD40^{-/-} and Act1^{-/-}BAFF^{-/-} mice had much reduced autoimmune diseases as compared to Act1^{-/-} mice, suggesting that Act1-regulated CD40- and BAFF-mediated pathways exert important impact on the regulation of autoimmunity. While the Act1^{-/-} mice represent a new mouse model for autoimmune diseases with intrinsic defects in B cell functions, we recently discovered that Act1 contains a U-box domain, belonging to the new U-box E3 ubiquitin ligase family. Act1 specifically ubiquitinates TRAFs (2, 3 and 6) and induces their proteasomal degradation. While CD40L stimulation leads to ubiquitination and degradation of TRAF2, 3 and 6, the degradation of TRAFs is greatly reduced in Act1^{-/-} primary B cells. While

the U-box domain alone from Act1 carries sufficient E3 activity, deletion of the B-box abolished the E3 activity of Act1, indicating that the U-box is the functional domain. Taken together, our results indicate that Act1 functions as an E3 ubiquitin ligase mediating the degradation of signaling molecules such as TRAFs thereby regulating B cell functions and autoimmunity.

01-17/P**INVOLVEMENT OF TUMOR NECROSIS FACTOR RECEPTOR-ASSOCIATED FACTORS 2, 3, 5 AND 6 IN VIRUS-ACTIVATED CYTOKINE EXPRESSION****Jensen SB¹, Paludan SR¹***¹Institute of Medical Microbiology and Immunology, University of Aarhus, Aarhus, Denmark.*

Macrophages reside in a broad variety of tissues throughout mammalian organisms and possess a pivotal role in the host's recognition of pathogens and the following initiation of the immune response by various mechanisms including cytokine secretion. Host cells utilize a group of germ line encoded receptors known as pathogen recognition receptors (PRR)s to detect pathogens including viruses. Subsequent to PRR activation intracellular signalling takes place leading to activation of gene transcription followed by expression of cytokines. The tumor necrosis factor receptor-associated factors (TRAF)s family of proteins has been shown to be involved in the diversification of PRR-induced transcription factor activation and cytokine expression. The aim of the present study is to characterize the roles of TRAF2, 3, 5 and 6 in virus-activated signal transduction and cytokine expression in a murine macrophage cell line (RAW264.7). The results obtained so far show that inhibition of either TRAF2 or TRAF6 by over-expression of dominant negative mutants impairs herpes simplex virus (HSV)-induced production of Interleukin 6 and tumor necrosis factor- α , whereas preliminary data suggest that RANTES expression is dependent on TRAF2 but less on TRAF6. Furthermore, blocking TRAF6 prevents HSV from activating nuclear factor- κ B. We are currently including the mitogen-activated protein kinase and interferon regulatory factor pathways in our investigations. The data presented at the meeting will also include TRAF3 and 5.

01-18/P**TLR9 AND RIG-I CO-ORDINATELY REGULATE CYTOKINE EXPRESSION IN RESPONSE TO HERPES SIMPLEX VIRUS TYPE 2 INFECTION****Rasmussen SB¹, Paludan SR¹***¹Inst. of Medical Microbiology and Immunology, University of Aarhus, Aarhus, Denmark*

During viral infections, different pattern recognition receptors (PRR)s become activated and initiate signalling pathways in the infected cells. Three of these pathways with important functions in antiviral defense lead to activation of interferon (IFN) regulatory factor (IRF), nuclear factor κ B (NF- κ B), and the mitogen activating protein kinases (MAPK)s, respectively. These pathways regulate expression of cytokines, chemokines, and IFNs. The aim of the present study is to characterize the requirement for different PRRs in the expression of cytokines during infection with the DNA virus herpes simplex virus (HSV)-2 in macrophages. Toll like receptor (TLR) 9 and retinoic-acid inducible gene I (RIG-I), detects unmethylated dsDNA and dsRNA, respectively. RIG-I and TLR9 were expressed in a dominant negative form and inhibited by an antagonist, respectively, and cytokines were measured after HSV-2 infection. We found that HSV-induced expression of RANTES, IL-6, and IFN- α/β was dependent on both RIG-I and TLR9, whereas TNF- α expression was dependent on only TLR9. Thus, apparently there is a differential and coordinated requirement for PRRs for induction of different cytokines during HSV-2 infection. In the ongoing part of the project we are examining how blockage of TLR9 or RIG-I affects the ability of HSV-2 to activate the IRF, NF- κ B, and MAPK pathways, and hence provide a mechanistic explanation for the observed requirement of PRRs for cytokine expression.

01-20/P

**THE PROINFLAMMATORY CYTOKINE-INDUCED
MGBP-2 INHIBITS CELL SPREADING ON FIBRONECTIN
BY INHIBITING RAC ACTIVATION**

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Interferon- γ (IFN- γ) treatment inhibits the ability of cells to spread on fibronectin. Ectopic expression of the GTPase, mGBP-2, a protein not expressed in most cells prior to interferon- γ treatment, is itself sufficient to retard spreading. The carboxy terminal α -helices of mGBP-2, completely lacking the GTP binding domain, can inhibit cell spreading as efficiently as wild type mGBP-2. However, isoprenylation of mGBP-2 is required to inhibit cell spreading, as a single amino acid change that prevents isoprenylation also abolishes the ability to inhibit cell spreading. These data suggest that lipid modification is required for correct intracellular targeting and that inhibition of spreading proceeds via one or more protein interactions with the carboxy terminal helices of mGBP-2. mGBP-2 inhibits Rac activation in cells spreading on fibronectin. Rac is a important regulator of the cytoskeletal changes that accompany cell spreading. Constitutively active Rac1(115I) is capable of rescuing the inhibition of spreading mediated by mGBP-2, but active forms of RhoA(63L) or Cdc42(V12) are not. This is the first report of a novel mechanism by which IFN- γ can alter the consequences of cellular interactions with extracellular matrix components. IFN- γ reduces cell spreading through the induction of the GTPase, mGBP-2. In turn, mGBP-2 alters cell spreading by interacting via its C-terminus to inhibit the integrin-mediated activation of Rac.

01-21/P

**GRANULOCYTE COLONY STIMULATING FACTOR
ACTIVATES DIFFERENT PATHWAYS AFTER BINDING
TO RECEPTORS IN TROPHOBLASTIC CELLS**

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The granulocyte colony-stimulating factor (G-CSF) is a cytokine that supports the proliferation and differentiation of myeloid precursors to neutrophils in bone marrow and activates the function of mature neutrophils. Although G-CSF functions are well known in granulocyte cell lines, receptors for this cytokine have also been found in non-haemopoietic sites, including endothelial cells, cardiomyocytes, neuronal precursors and placenta. In a previous work we identified the cytokine conformational epitope involved in binding to receptors from placenta and myeloid cells (NFS-60). In the present study, in order to explore the biological action of G-CSF in placenta, we employed the trophoblastic cell line JEG-3. We first revealed the presence of cytokine specific receptors in these cells and determined affinity constant and binding capacity values of $3 \times 10^9 \text{ M}^{-1}$ and 1290 sites/cell, respectively, which were similar to those obtained in NFS-60 cells. Since G-CSF binding to hematopoietic cells receptors induces signal transduction by activating the receptor-associated Janus family tyrosine kinases (Jak) and signal transducer and activator of transcription (STAT) proteins as well as the mitogen activated protein kinases (MAPK), we then examined the possible contribution of these pathways in trophoblastic cells signalling. Results showed that G-CSF induced Jak1, Jak2, Tyk-2 and STAT3, but not STAT1 and STAT5 phosphorylation in both myeloid and trophoblastic cells. Furthermore, p38 MAPK and p44/42 MAPK were also phosphorylated in a time-dependent manner. Although G-CSF induces a mitogenic response on NFS-60 cells, trophoblastic cells proliferation was not stimulated in the presence of cytokine. However, when the effect of G-CSF on cellular viability was evaluated, it was shown that cytokine-stimulated JEG-3 cells were protected from cell death induced in the absence of serum. In summary, our results represent the first report of intracellular signals triggered after G-CSF binding to receptors from placenta and suggest a functional role of G-CSF in trophoblastic cells, probably related to cell survival.

01-22/O

**HUMAN INTERFERON ALPHAS: FUNCTIONAL ANALYSIS
USING GENE EXPRESSION MICROARRAYS AND
PROTEOMICS**

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Our studies focus on how human interferon-alphas (IFN-alphas) elicit their biological (antiviral, antiproliferative and immunomodulatory) activities. To determine the domains of the IFN-alphas that are important for antiviral and antiproliferative activities we genetically engineered, expressed and purified IFN-alpha hybrids and mutants derived from human IFN-alpha 21b and IFN-alpha 2c. In an effort to better understand the mechanisms of action and signaling pathways of the IFN-alphas, gene expression microarray and proteomic studies were performed in Daudi cells. It was hypothesized that these two technologies might provide insight into which pathways contribute to the different levels of antiproliferative and antiviral activities observed with the various IFN-alpha hybrids and mutants. Oligonucleotide gene expression microarray analysis showed that there were distinct expression patterns corresponding to the type of IFN-alpha. Real-time PCR was used to confirm a number of the changes that were observed by microarray analyses. These diversities in gene regulation may contribute to different biological activities observed in the studied IFNs. Quantitative proteomic analysis of Daudi cells treated with different interferon-alphas (IFN-alpha 2c and IFN-alpha 21b) using Isotope-Coded Affinity Tag (ICAT) reagents and mass spectrometry was performed. We compared the differentially regulated proteins of Daudi cells treated with IFN-alpha-2c and IFN-alpha 21b for 24 hours to the microarray data and found many of the changes to be similar. Comparative reverse-phase microarray proteomic analysis was done to validate some of the changes observed from the ICAT analyses. The diversity in gene regulation revealed by these studies may contribute to the different biological activities observed in the studied IFN-alphas.

01-23/P

**ENLARGED SPLEENS WITHOUT ENLARGED LYMPH
NODES IN *TLR3^{-/-}PKR^{-/-}* MICE**

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Both Toll-Like Receptor 3 (TLR3) and double stranded RNA-activated Protein Kinase R (PKR) have been identified as receptors for double stranded RNA (dsRNA). Various defects in response to dsRNA have been characterized in cells from *tlr3^{-/-}* and *pkr^{-/-}* mice. However to date the extent of redundancy in these pathways and their relative contributions to dsRNA signaling and viral defense have been unclear. In order to more clearly distinguish the roles of TLR3 and PKR in dsRNA signaling and anti-viral defense we generated *tlr3^{-/-}pkr^{-/-}* mice. Initial analysis of hemopoietic organs revealed no difference from wild-type controls in the proportions of CD4⁺ and CD8⁺ T cells, B cells, granulocytes, macrophages, natural killer cells or CD11c⁺ dendritic cells in spleen or bone marrow in *tlr3^{-/-}*, *pkr^{-/-}* and *tlr3^{-/-}pkr^{-/-}* mice. Unexpectedly a subset of mice derived from a single *tlr3^{-/-}pkr^{-/-}* breeding pair developed enlarged cervical, axillary and inguinal lymph nodes between 12 and 16 weeks of age. *tlr3^{-/-}* mice developed enlarged lymph nodes at a higher frequency than wild-type, *pkr^{-/-}* or *tlr3^{-/-}pkr^{-/-}* mice. Mice with enlarged lymph nodes always showed enlarged spleens. By 16 weeks enlarged spleen and lymph nodes showed lymphocyte infiltration and lacked normal organ architecture. This phenotype may be the consequence of an as-yet uncharacterized genetic lesion resulting in hyperplasia or autoimmunity. The role of an infectious agent in this process also remains to be investigated.

01-24/O

IL-7 IN THE LIFE, DEATH, PROLIFERATION AND DIFFERENTIATION OF T CELLS.**Scott K. Durum, Wenqing Li, Qiong Jiang, Annette R. Khaled.***Laboratory of Molecular Immunoregulation, CCR, NCI, NIH, Frederick, MD.*

IL-7 is required by T cells at two stages, first as immature thymocytes, then later as mature T cells in peripheral lymphoid organs. Part of the requirement for IL-7 is to block apoptosis. We observed that this anti-apoptotic effect is partly achieved by inducing synthesis of Bcl-2, which protects mitochondria. IL-7 also protects the T cell from activation of the death proteins Bax, Bad and Bim which are post-translationally activated by different mechanisms, to be discussed. A second requirement for IL-7 is to induce cell division which we observe occurs by two mechanisms that relieve G1 arrest. One cell cycle mechanism occurs through protecting the phosphatase Cdc25a from proteosomal degradation via a novel pathway to be discussed. Cdc25a removes an inhibitory phosphate from Cdk2, resulting in phosphorylation of Rb and entry into S phase. A second mechanism is by inducing proteosomal degradation of p27, an inhibitor of Cdk4; this occurs through a novel mechanism to be discussed. Because we observed that several other cytokine receptors, in addition to IL-7 receptor, can also induce cell survival and proliferation by similar pathways, we asked whether the IL-7 receptor intracellular domain delivers unique signals in T cell development. We observed that the IL-9 receptor intracellular domain can replace that of the IL-7 receptor in development of $\alpha\beta$ T cells. This suggests that the survival and proliferative functions of IL-7 receptor are not unique, although we do not understand them completely. However IL-7 also induces rearrangement of the TCR γ locus and development of $\gamma\delta$ T cells, and this activity could not be replaced by IL-9 receptor suggesting that IL-7 receptor also delivers unique signals.

01-25/P

SIGNALING PATHWAYS MEDIATING LPS-INDUCED STABILIZATION OF FPR1 MRNA**Mandal P, Hamilton T***Department of Immunology, Lerner Research Institute, Cleveland Clinic Foundation*

LPS promotes the expression of the formyl peptide receptor 1 (FPR1) gene in leukocytes in part via prolonging the half life of the mRNA. In order to identify the TLR4-initiated signaling events that couple to FPR1 mRNA stabilization, macrophages were treated with LPS for 4 hrs in the absence or presence of a selection of pharmacologic inhibitors targeting a number of known signaling pathways. While inhibitors of protein tyrosine kinases, MAP kinases, and stress activated kinases had only modest effects on the response to LPS, the blockade of NF κ B activation via the IKK inhibitor parthenolide completely prevented the elevation of FPR1 mRNA. The PI3 kinase inhibitor LY294002 (LY2) was also a potent inhibitor. While inhibition of NF κ B activation blocked expression of both FPR1 and the LPS-induced neutrophil chemoattractant CXCL1 (KC) mRNAs, the effect of LY2 was selective for FPR1. Though LY2 was demonstrated to be a potent inhibitor of PI3 kinase activity, a structural analogue of LY2 (LY303511 or LY3) that did not inhibit PI3 kinase was equally effective at preventing LPS-stimulated FPR1 expression. LY2/LY3 blocked neither the activation of NF κ B nor the expression of KC mRNA. Furthermore, LY2/LY3 could block LPS-induced mRNA stabilization of FPR1 even when added 4 hrs after LPS while the NF κ B inhibitor parthenolide had no effect on the rate of FPR1 mRNA decay. These findings demonstrate that LY2/LY3 targets a novel TLR4-linked signaling pathway that selectively couples to the stabilization of FPR1 mRNA.

01-26/P

SIGNALING AND CYTOKINE PROFILING IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS WITH BIO-PLEX SUSPENSION ARRAY SYSTEM**Zhu-Shimoni J, Zimmerman R, Zhou H, Asakawa K, Mariano M, Allauzen S***Life Science Group, Bio-Rad Laboratories, Hercules, California, USA*

Cytokines, originally characterized only as leukocyte effectors, are now recognized as pleiotropic messengers produced by and acting on virtually every known cell type. These soluble proteins mobilize specific cellular receptors, thereby regulating diverse functions in cell development, activation, and metabolism. While cytokines are critical for normal cellular functions, aberrant cytokine production has been associated with diseases and infectivity. Recent studies have reported that the effects of cytokines are mediated by different stress- and mitogen-induced protein (MAP) kinases. These kinases conduct signaling transduction through an array of events of phosphorylation that eventually lead to activation or inactivation of cellular responses including apoptosis, cell growth and proliferation. Thus, cytokine monitoring has become an increasingly important component of drug development, particularly in oncology, immunotoxicity, autoimmunity, and infectivity. At the same time, assessing phosphorylation status of kinases and their substrates as well as transcription factors becoming equally critical for unraveling the mechanisms of gene expression and regulation. Human peripheral blood mononuclear cells were treated with different cytokine and/or mitogens: IL-10, Lipopolysaccharide (LPS), Pokeweed lectin mitogen (PWM) and IL-10 in combination with LPS. The samples of supernatant were collected and tested for secreted cytokine levels. Mononucleocytes were harvested and analyzed for phosphoprotein as well as intra cellular cytokines. Using Bio-Plex suspension array system we demonstrate profiling the levels of 50 cytokines and chemokines, both secreted and also intracellular, and 25 phosphoproteins in response to mitogen stimulation. Cytokine and signaling profiles of mononucleocytes responding to mitogens display capability of using this array system to examine different mechanisms of immunoregulations, as well as signaling transduction which establishes the correlation between mitogen response and molecular profiling. The approach may complement with other methods in the rapid screening for and identification disease related biomarkers, biological contaminants and environmental pathogens, or in drug development such as pharmacokinetic/pharmacodynamic studies.

01-27/P

ADAPTIVE IMMUNE RESPONSE IS IMPAIRED IN TYROSINE KINASE 2 DEFICIENT MICE**Simme O¹, Sexl V¹, Stoiber D¹***¹Institute of Pharmacology, Center of Biomolecular Medicine and Pharmacology, Medical University of Vienna, Austria*

The Janus Kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs) are important mediators of cytokine signaling. Multiple aspects of haematopoiesis, such as haematopoietic cell survival, differentiation and proliferation, are dependent on this signalling pathway. Recently our group has identified a role of the JAK family member Tyrosine Kinase 2 (TYK2) in tumour surveillance. TYK2 deficient mice are more prone to develop Abelson induced tumours than wildtype animals. It could be shown that the cytolytic cells of the innate immune system, the natural killer NK cells, are impaired in TYK2 knockout mice. Since TYK2 has an established role in type 1 and type 2 interferon, as well as interleukin-12 signalling, we postulated an involvement of this kinase in the generation of adaptive immune responses. So far, *in vivo* CTL assays have indeed revealed a defect in antigen-specific cytotoxicity in the absence of TYK2. The induction of an adaptive immune response requires efficient stimulation and activation of immature dendritic cells (DCs) and the subsequent activation, proliferation and differentiation of naive T cells into functional effector cells. The focus of our study is the identification of the step(s) in this chain of events that is responsible for the observed defective CTL response.

01-28/P

REGULATION OF INTERLEUKIN-6-SIGNAL TRANSDUCTION BY THE ADENYLATE CYCLASE PATHWAY

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Many recent studies focussed on the negative regulation of interleukin-6 (IL-6) signal transduction through the IL-6-induced feedback inhibitors suppressors of cytokine signalling (SOCS)1 and SOCS3 or protein tyrosine phosphatases such as SHP2 and TcPTP. In addition, studies on the crosstalk between pro-inflammatory mediators (interleukin-1, tumor necrosis factor, lipopolysaccharid) and interleukin-6 elucidated further regulatory mechanisms, such as competition of transcription factors for binding to overlapping binding sites within the promoters of IL-6-inducible genes or stabilization of mRNAs of IL-6-target genes. Less is known about the regulation of IL-6 signal transduction by hormones/cytokines signalling through G-protein-coupled receptors. This is particularly astonishing since many of these hormones (such as prostaglandins and chemokines) play an important role in inflammatory processes. We set up a study to analyse whether signalling through G-protein-coupled receptors has a regulatory function on IL-6-induced signal transduction. Adenylate cyclase is a downstream target of activated G-protein-coupled receptors. Both, activation of adenylate cyclase or a block of its inhibition counteracted IL-6-induced ERK-activation but not STAT3-phosphorylation through a PKA- and Src-dependent mechanism in murine embryonal fibroblasts as well as in human primary dermal fibroblasts. The same observation was made in fibroblasts stimulated with adenylate cyclase activating prostaglandin. In summary, our data uncovered a specific crosstalk between the adenylate cyclase cascade activated through G-protein-coupled receptors and the IL-6-induced MAPK cascade.

01-29/P

REGULATION OF STABILITY AND FATE OF THE TRIM8/SOCS-1 COMPLEXES BY SERINE/THREONINE KINASE ACTIVITY

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Members of the suppressor of cytokine signalling (SOCS) family of signalling molecules regulate the cytokine-mediated biological response. In particular, SOCS protein stability seems to depend on proteosomal activity and ubiquitination of targeted complexes. In addition, SOCS-1 interacts with TRIM8/GERP, a new RING finger protein, whose expression can be induced by interferon-gamma in epithelial and by IL-4 on lymphoid cells. Co-expression of TRIM8/GERP with SOCS-1 decreases SOCS-1 protein stability and levels as suggested by cyclohexamide treatment of TRIM-8/SOCS-1 transfected cells. On this report we show that TRIM-8 can physically complex either SOCS-1 or Pim-2 in vitro suggesting the development of a trimetric complex whose stability depends on the level of SOCS-1 and TRIM-8 phosphorylation by Pim-2. Co-expression of TRIM-8/SOCS-1 and Pim-2 greatly reduces the stability of SOCS-1 induced by Pim-2 and attenuates its ability to inhibit an IFN- γ mediated transcription activity using a GAS-Luciferase transfection assay. Taken together these data suggest that TRIM8/GERP may be a main regulator of SOCS-1 function through kinase activity of selected Pim family members.

01-30/P

A P38-DEPENDENT PATHWAY REGULATING ACTIVATION OF DENDRITIC CELLS DURING VIRAL INFECTION THROUGH A POST-TRANSCRIPTIONAL MECHANISM

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Dendritic cells (DCs) play a central role in regulation of the innate and adaptive immune system via expression of cytokines (e.g. Interleukin (IL)-12 and interferon (IFN)- α/β) and presentation of antigen to naïve T cells in lymphoid organs. DCs recognise pathogens through pattern recognition receptors and transduce intracellular signals leading to cell activation. Here we show, that activation of DCs during viral infection is regulated through a signalling pathway involving the MAP kinase p38, and that this pathway regulates expression of cytokines at the post-transcriptional level. Specifically, we show that while both infectious and UV-inactivated Sendai virus activated signal transduction to NF- κ B and accumulation of IL-12 and IFN- α/β mRNAs, only infectious virus was able to allow production of the cytokines. Furthermore inhibition of p38, which was activated by infectious but not UV-inactivated virus, abrogated production of IL-12 by virus-infected cells, but allows cytokine mRNAs to accumulate. The observed phenomenon was not specific to Sendai virus infection, since a similar picture was observed when DCs were challenged with other viruses. Thus, the data suggest that a p38-dependent pathway regulates DC activation at the post-transcriptional level during viral infections.

01-31/O

SLY1 AND ITS ROLE IN ANTIGEN RECEPTOR DRIVEN T CELL ACTIVATION

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Lymphocyte activation by antigen receptor triggering is an essential step in adaptive immunity which must be tightly regulated to mount an immune response towards a pathogen on one side and to avoid autoimmune disorders on the other. An increasing number of molecules involved in the signal transduction pathway downstream of T or B cell receptors has been discovered in the last years. Major players in this complex network are adapter proteins containing characteristic SH2 or SH3 domains which are known to mediate protein protein interactions. SH3 and SAM domain containing protein expressed in lymphocytes (SLY) is a member of a recently identified family of putative adapter and/or scaffold proteins highly conserved in mammals. SLY is exclusively expressed in lymphocytes and has been shown to be phosphorylated specifically upon antigen receptor engagement.

To investigate the physiological functions of SLY, we generated *sly*-mutant mice expressing a truncated protein lacking the nuclear localisation signal and the phosphorylation site (SLY1^d). *Sly*^{1d/d} mice exhibit reduced lymphoid organ sizes, diminished marginal zone B cell numbers and severely impaired antibody responses against T-dependent and -independent antigens. B and T cell proliferation is attenuated and T cell cytokine production is severely reduced. *In vivo*, survival of semi-identical cardiac allografts was substantially prolonged in *Sly*^{1d/d} mice. However, global tyrosine and MAP Kinase phosphorylation, Ca²⁺ flux and transcription factor activation are normal. We show that SLY wild-type protein specifically shuttles in between nucleus and cytoplasm after antigen receptor activation. In contrast, SLY-mutant

protein shuttling is impaired. The signal transduction pathway leading to the induced translocation of SLY protein and the role of the phosphorylation status, the nuclear localisation signal and the SH3 and SAM domains are further dissected. A new protein playing a non-redundant role needed for full activation of lymphocytes *in vitro* and *in vivo* has been identified.

01-32/P

UBIQUITIN-LIKE DOMAIN IN TANK BINDING KINASE 1 IS INVOLVED IN THE REGULATION OF IFN REGULATORY FACTOR-ACTIVATION

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TANK Binding Kinase 1 (TBK1) is known to play an important role in the regulation of activation of its downstream transcription factors, interferon regulatory factor 3 (IRF3) and IRF7. TBK1 is activated by various stimuli, including lipopolysaccharide or dsRNA virus-infection via Toll-like Receptors. TBK1 directly phosphorylates IRF3 and IRF7, which bind to the IFN-stimulated response element (ISRE) motifs and upregulate IFN-inducible genes. However, the precise molecular mechanism in which TBK1 regulates activation of these molecules is not elucidated. We have recently identified the novel ubiquitin-like domain (UBL) in TBK1 and showed to play a role in the modulation of kinase domain of TBK1 and the regulation of IRF activation. We constructed deletion-UBL mutant (Δ -UBL) as well as mutations in the hydrophobic patch of UBL in TBK1 and demonstrated that both Δ -UBL and hydrophobic patch mutants completely abolished its potential to induce IFN- β and RANTES-promoter activities. Moreover, biochemical studies indicated that UBL is engaged in the intra-molecular binding with the amino-terminal part of TBK1 thus modulating its protein kinase activity. We also found that UBL plays a role in the recruitment of IRFs to TBK1 leading to their subsequent phosphorylation. In conclusion, we provide a new evidence for the role of UBL domain in the regulation of intra-molecular folding of TBK1, recruitment of IRFs and regulation of IFN-inducible genes.

01-33/P

GENOME OLIGO MICROARRAY ANALYSIS OF DAUDI CELLS TREATED WITH INTERFERON- α 2C AND INTERFERON- α 21B

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NIAID/NIH

Genome microarray technology whereby oligonucleotides are arrayed onto glass slides, possesses the ability to take a molecular snapshot of a cell at any given moment, so that relative levels of mRNA between two samples of interest can be calculated. In this study, ~21K 70mer oligonucleotides (Operon Biotechnologies, Huntsville, AL) were spotted onto epoxy coated glass slides and used to examine the transcriptome of Daudi (Burkitt's lymphoma) cells that were treated with recombinant IFN- α 2c or IFN- α 21b for a period of 24 hours. Untreated Daudi cells were also collected at 24 hours as control. Following reverse transcription and indirect labeling with either Cy3 or Cy5, samples were mixed and hybridized onto arrays. After overnight hybridization, arrays were scanned and images collected using an Axon GenePix 4200A scanner along with GenePix Pro image analysis software (Molecular Devices, Sunnyvale, CA). Technical array replicates were performed for both the IFN- α 2c and IFN- α 21b treatments (5 arrays and 4 arrays respectively in total). Array data was then uploaded to *mAdb* (NCI/CCR, Bethesda, MD) where it was analyzed using various bioinformatics tools. Interesting to note is that we found 395 common genes between the IFN- α 2c and IFN- α 21b treatments using a very stringent 2 fold cutoff. Among these common transcript changes are many interferon inducible genes, along with the down-regulation of genes known to play a role in cell proliferation. By employing a t-test strategy, genes that were uniquely over or under expressed between the two different interferon treatments were obtained. In an effort to confirm these results, quantitative real-time RT-PCR has been and will continue to be performed.

01-34/P

PROTEOMIC ANALYSIS OF LYSATES FROM DAUDI CELLS TREATED WITH INTERFERON α 2C OR INTERFERON- α 21B

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The field of proteomics has provided a platform to study the protein expression of an organism under defined conditions. Specifically, characterization can be expanded to include protein identification, relative quantitation, modifications (e.g. phosphorylation, glycosylation) and protein-protein interactions. In this study we focus on the Daudi (Burkitt's lymphoma) cell proteome after treatment with recombinant IFN- α 2c or IFN- α 21b for a period of 24 hours, using untreated cells as a control. Utilizing isotope coded affinity tag (ICAT) technology, proteins were labeled with either the light (¹³C₀) or the heavy (¹³C₉) version of the cleavable ICAT reagent. The samples were then digested and the cICAT-labeled peptides were isolated using immobilized avidin chromatography and fractionated using strong cation exchange (SCX) chromatography. Individual fractions were analyzed by reversed-phase microcapillary liquid chromatography (μ RPLC), coupled online with an ion trap MS (LCQ Deca XP, Thermo Finnigan, San Jose, CA) operated in a data-dependent MS/MS mode. Peptides were identified and quantified using commercially available software packages. Of those proteins identified by two or more peptides and having an abundance cutoff above or below two fold, 83 proteins were up-regulated and 38 proteins were down-regulated compared to untreated cells in IFN- α 2c treated cells, whereas in IFN- α 21b treated cells, 3 proteins were up-regulated and 35 proteins were down-regulated compared to untreated cells. These values represent total protein and were validated by both reverse phase protein array using antibodies directed against the proteins of interest as well as Western Blot analysis.

01-35/P

THE PROLYL ISOMERASE PIN1 REGULATES THE TRANSCRIPTIONAL ACTIVITY OF NF- κ B TARGET GENES IN GLIOBLASTOMA

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Glioblastoma (GBM) is the most frequent and malignant primary brain tumor in adults. While new therapeutic regimens have marginally improved overall patient lifespans, the lethality and aggressive nature of GBM warrants further investigation into its underlying biology. The mammalian NF- κ B family includes five members: p65 (RelA), RelB, c-Rel, p50/p105 and p52/p100. In the unstimulated state, these proteins exist in the cytoplasm bound to the I κ B inhibitory complex. Upon stimulation, I κ B is degraded and NF- κ B translocate to the nucleus where they activate a number of pro-inflammatory target genes, such as IL-8 and MMP-9. The NF- κ B pathway, particularly activation of p65, is aberrantly activated in GBMs. A protein that recently has been shown to play a regulatory role in the NF- κ B pathway is peptidyl-prolyl isomerase (PPIase) Pin1. Pin1 is the only member of the PPIase family which has been shown to preferentially bind phosphorylated serine or threonine residues immediately preceding proline residues (pSer/Thr-Pro) and promote isomerization of this bond. We hypothesize that Pin1 may retain activated p65 in the nucleus, fostering increases in NF- κ B signaling. To study the role of Pin1 in NF- κ B signaling, we have successfully created a stable, tet-inducible siRNA cell line that specifically knocks down Pin1 expression. Our studies and others have demonstrated elevated levels of Pin1 protein in human GBM samples and tumor-derived cell lines. We have also shown protein-protein interaction between p65 and Pin1 via coimmunoprecipitation and immunofluorescence. Finally, we have seen significant inhibition of IL-8 and MMP-9 promoter activity in the absence of Pin1, indicating a functional effect of Pin1 on NF- κ B signaling. The current focus of our studies is to further investigate the effects of Pin1-p65 interaction on these and other NF- κ B regulated genes to

discover the specific transcriptional programs of these genes in the presence and absence of Pin1.

01-36/O

TYPE I AND II IFN-INDUCED ACTIVATION OF MTOR-DEPENDENT SIGNALING PATHWAYS AND REGULATION OF MRNA TRANSLATION

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The interferon (IFN) signals required for initiation of mRNA translation and ultimately induction of protein products that mediate IFN-responses remain unknown. We have previously shown that Type I and II IFNs induce activation of the p70 S6 kinase and phosphorylation of the translational repressor 4E-BP1, in a PI3K- and mTOR-dependent manner. In the present study we examined the functional relevance of activation of this pathway using knockout MEFs for various known signaling elements of this cascade. Our data demonstrate that Type I IFN-inducible expression of ISG15 protein and Type II IFN-inducible expression of IP-10 protein, as well as generation of Type I IFN-dependent antiviral responses are defective in MEFs with targeted disruption of both the Akt1 and Akt2 genes (Akt1^{-/-}Akt2^{-/-}). Similarly, overexpression of a dominant-negative Akt protein in cells that express endogenous Akt results in decreased ISG15 protein expression in response to IFN α , suggesting an important positive regulatory role for this kinase in the induction of IFN-responses. On the other hand, Type I IFN-inducible ISG15 protein and Type II IFN-inducible IP-10 expression is enhanced in 4E-BP1 and TSC2 knockout cells. Similar regulatory effects on ISG15 protein expression were also observed in cells in which endogenous expression of 4E-BP1 was blocked using specific siRNA. Moreover, the induction of Type I IFN-dependent antiviral responses is enhanced in cells with targeted disruption of the 4E-BP1 and TSC2 genes, underscoring the functional relevance of these effectors of the IFN-activated mTOR pathway in the initiation of mRNA translation. Altogether, our studies establish a critical role for mTOR-dependent pathways in IFN-dependent mRNA translation, and identify Akt, 4E-BP1, and TSC2 as key regulators of the induction of IFN-dependent biological responses.

01-37/P

A TIME-AND DOSE-DEPENDENT STAT1 EXPRESSION SYSTEM

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The signal transducer and activator of transcription (STAT) family members mediate a variety of cytokine dependent gene regulations. STAT1 has been mainly characterized by its role in interferon (IFN) type I and II signaling and STAT1 deficiency leads to high susceptibility to several pathogens. We established a dimerizer inducible system for fine-tuned analysis of STAT1 function and demonstrate the functionality in mouse embryonal fibroblasts and embryonic stem cells. We show that the two-vector based system is highly inducible and does not show any STAT1 expression in the absence of inducer. Reconstitution of STAT1-deficient cells with inducible STAT1 restores IFN γ mediated gene induction, antiviral responses and STAT1 activation remains dependent on cytokine stimulation. STAT1 expression is induced rapidly upon addition of dimerizer and expression levels can be regulated in a dose-dependent manner. Furthermore we provide

evidence that the system is suitable for the use in transgenic mice, although further optimization will be required. To our knowledge this is the first report for inducible STAT1 expression providing a powerful tool for the analysis time- and dose-dependent STAT1 functions.

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01-38/P

DISSECTION OF KINASE-DEPENDENT AND -INDEPENDENT FUNCTIONS OF MURINE TYK2

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Previous work with mutant cell lines and knock-out mice has established the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway as central requirement for signalling transcriptional responses for a number of cytokines and growth factors. Deregulated catalytic activity of mammalian JAKs (Jak1-3, Tyk2) has been implicated in many diseases, including chronic inflammation and cancer. Additionally, there is increasing evidence for JAK functions distinct from their tyrosine kinase activity. We constructed a kinase-inactive murine Tyk2 (Tyk2K923E) by mutating the invariant lysine in the ATP binding site of the kinase domain. Analysis of stably transfected murine fibroblast cell lines revealed a dose-dependent, receptor-specific dominant negative effect of Tyk2K923E. IFN β -induced formation of Stat1- and Stat3- homo- and heterodimers and Stat5 activation were strongly inhibited by Tyk2K923E, whereas ISGF3 activation was only modestly affected. In contrast, Stat activation upon IL-6 stimulation was not altered by the presence of kinase-inactive Tyk2. Consistent with previous reports using human cell lines, IFN β -mediated activation of Akt/PKB is Tyk2-dependent in our system and preliminary data suggest that this function is at least partially kinase-independent. Microarray analysis identified genes regulated by IFN β in a Tyk2-dependent manner. Importantly, Tyk2 deficiency does not just result in reduced activation of IFN β responsive genes, but has a differential effect on a specific set of genes. Furthermore, expression of kinase-inactive Tyk2 does not mimic Tyk2 deficiency, but has a rather complex effect on IFN β mediated gene regulation. Tyk2 is involved in signalling via a number of cytokines whose cognate receptors are expressed in a cell type or differentiation state dependent manner. Generation of Tyk2K923E knock-in mice will allow us to study Tyk2 kinase-independent functions in complex immunological situations and to address aspects of cell type specificity in cytokine responses. ES cell targeting has been successfully performed and chimeric mice are currently tested for germ-line transmission.

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01-39/P

IN T LYMPHOCYTES, ABLATION OF STAT3 EXPRESSION REINSTATES STAT1-DEPENDENT APOPTOSIS BY IFN γ , IL-6 AND IFN α

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The Interferon gamma (IFN γ)/Signal Transducer and Activator of Transcription (STAT) 1 pathway is mainly involved in the control of T lymphocytes homeostasis. However, through different mechanisms, normal resting and neoplastic T cells become resistant to its antiproliferative effect. STAT3 is a transcriptional factor activated by many cytokines including IFN γ , important in favoring proliferation and protecting from apoptosis. Here, we investigate STAT3 role in the negative regulation of the IFN γ /STAT1 apoptotic pathway, by small interfering RNA-mediated inhibition of STAT3 expression in a human malignant T cell line, ST4. In cells expressing normal STAT3 amounts either IFN γ or IL-6 induced a weak activation of STAT1, whereas in the absence of STAT3 both IFN γ - and IL-6-dependent STAT1 activation was enhanced. Of note, in the absence of STAT3 the ability to induce MHC class I expression was maintained by IFNs and markedly enhanced by IL-6. IFN α induced a strong STAT1 activation independently on the presence or the absence of STAT3. Analyzing the apoptotic response, IFN γ , IL-6 and, to a high degree, IFN α switched on the apoptotic program in T cells devoid of STAT3, whereas they were ineffective in cells expressing normal amounts of STAT3. In conclusion, in human malignant T lymphocytes, the inhibition of STAT3 expression resulted in the reinstatement of IFN γ and IL-6 induced-STAT1 activation, MHC class I induction and apoptosis. Moreover these data also indicated that the IFN α -dependent apoptosis is greatly enhanced by the ablation of STAT3 expression. We are currently performing experiments in SCID mice inoculated with ST4 cells expressing or not STAT3, to assess whether IFN γ , IFN α and/or IL-6 can inhibit the growth of tumor cells also *in vivo*.

01-41/P

INTERLEUKIN-27 SIGNAL TRANSDUCTION IN THE LIVER

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The recently discovered cytokine Interleukin-27 (IL-27) belongs to the IL-6/IL-12-cytokine superfamily (Interleukin-6, OncostatinM, Leukemia Inhibitory Factor, Interleukin-31, Interleukin-11, Ciliary Neurotrophic Factor, Cardiotrophin-1, Cardiotrophin-like Cytokine, Interleukin-12 and Interleukin-23). It is a heterodimeric cytokine composed of the two subunits p28 and Epstein-Barr virus induced gene 3 (EBI-3). Interleukin-27 is secreted by activated antigen presenting cells and possesses pro- as well as anti-inflammatory properties. In the early phase of an infection it can induce the production of IFN- γ by CD4- and CD8-T cells whereas it suppresses excessive production of pro-inflammatory cytokines such as IL-1, IL-8 and IFN- γ in a later phase. Signal transduction of IL-27 occurs via a receptor complex containing the receptor chains gp130 and WSX-1. Although gp130 is ubiquitously expressed, WSX-1 is mainly presented on naïve T-cells, polarized Th-cells and NK cells. Nevertheless the receptor is also present on myeloid cells as well as on activated endothelial and epithelial cells. In the present study we investigated a possible function of IL-27 in the liver as previous studies analyzing the expression of WSX-1 in the liver lead to inconsistent results. We detected expression of WSX-1 in primary rat hepatocytes and in the human and rat hepatoma cell lines HepG2, Huh-7 and Fao. All three cell lines respond to IL-27, although to a different degree. IL-27 induced the activation of Janus kinases as well as the transcription factors STAT1 and STAT3. Further studies will deal with the possible involvement of other signaling components as well as with the characterization of target genes for IL-27 in the liver.

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01-42/P

JAK-1/SMAD SIGNALING DEPENDENT GENES EXPRESSION ASSOCIATED WITH THE NEUROGENIC TO ASTROGLOGENIC TRANSITION POINT OF BRAIN DEVELOPMENT

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As the crucial astrogliogenic pathway which could be modulated by variety of environmental factors, JAK/STAT cytokine signaling represents basically the path that has to be converted in order to transform reactive gliosis into purposeful repair reaction in a therapeutical approach of brain damage. Though loss of function studies have helped to clarify the role of some of its components like LIF or gp130, which turned out to be essential for astroglial differentiation, the role of JAK-1 molecule in this process remains elusive. It seems that depending on the preactivation level of JAK/STAT signaling at a given time point during development, depends the final outcome of the interactions among molecules basically defined astrogliogenic like these are the members of TGF- β superfamily. Therefore, to address the question on the role of JAK-1 molecule in TGF- β controlled sequential process of brain development, we have used two cell lines of nonneural origin: one that is deficient in JAK-1 molecule and the other overexpressing it. Upon their transfection with TGF- β receptor associated molecules. Flagged SMAD-3, SMAD-2, or SMAD-1, the expression pattern of astrogliogenic versus neurogenic molecules has been followed. The activation status of NF- κ B pathway which seems to be equally needed for both neuronal and astroglial differentiation was examined as well. We hope that by dissecting signaling events behind the process of reactive gliosis induced by all types of central nervous system injuries, will get better clue on how to approach the problem of a repair process in adult brain.

01-43/O

IMPORTANCE OF STAT1 SERINE PHOSPHORYLATION IN REGULATING PROLIFERATION, SURVIVAL AND ACTIVITY OF CELLS OF THE IMMUNE SYSTEM

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STAT1 (signal transducer and activator of transcription 1) is essential for reprogramming gene expression in response to type I and type II interferons. Activation of STAT1 by interferons causes it to dimerize, translocate to the nucleus, bind target promoters, and stimulate transcription. Activation is caused by two different phosphorylation events. Phosphorylation of tyrosine 701 triggers dimerization, nuclear translocation and promotor binding, whereas the phosphorylation of a C-terminal serine residue, S727, enhances transcriptional activity. To study the importance of S727 phosphorylation in the immune system, we generated gene-targeted mutant mice expressing a S727A mutant of Stat1.

CD4⁺ and CD8⁺ T cells derived from STAT1S727A mice showed an enhanced proliferative response to activating stimuli, which was in part due to reduced apoptosis of the proliferating cells. By contrast, an *in vivo* assay of peptide-specific cytotoxic T cell activity showed a defect of STAT1S727A mice to generate functional cytolytic T cells. Further studies suggested that this defect reflected an inability of STAT1S727A to function properly in the context of type I interferon (IFN) responses. In line with this assumption, dendritic cells derived from STAT1S727A mice displayed reduced responsiveness to type I IFN treatment *in vitro*. Together our data suggest an essential and non-redundant role of Stat1 serine phosphorylation in adaptive immune responses.

01-44/O

THE ROLE OF IRAK-4 KINASE ACTIVITY IN IL-1 β -MEDIATED SIGNALING

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Activation of interleukine-1 receptor (IL-1R) or toll like receptors (TLRs) by binding of pro-inflammatory cytokines or pathogen-associated molecular products leads to induction of cellular signaling pathways, involving a cascade of adaptor molecules, kinases and transcription factors, that control expression of inflammatory genes. IL-1R-associated kinases (IRAKs) are recruited to the receptor complex upon activation. IRAK-4 has been reported pivotal for IL-1R- and TLR-mediated signaling, but controversial data exist concerning its kinase function. To investigate the role of IRAK-4 kinase activity *in vivo*, 'knock-in' mice were generated by replacing the wild type (wt) IRAK-4 with a mutant gene encoding kinase inactive protein. Homozygous mutant mice (IRAK-4 KD) were viable and reproduced as wt mice. IL-1 β failed to activate the NF κ B, p38 and JNK pathways in embryonic fibroblasts (MEF) derived from IRAK-4 KD mice. Lipopolysaccharide (LPS)-stimulated bone marrow derived macrophages (BMDM) from IRAK-4 KD or IRAK-4^{-/-} mice also displayed reduced activation in JNK and p38 signaling, while activation of the NF κ B pathway was comparable to wt cells. In line with this finding, we observed early LPS-mediated induction of mRNAs coding for inflammatory proteins in both wt and mutated cell. In contrast to wt BMDM, expression of most LPS-induced mRNAs declined rapidly over time in IRAK-4 KD BMDM, resulting in significantly reduced production of the respective proteins. This finding suggests a role of IRAK-4 in late phases of LPS-mediated gene expression. To further elucidate the mechanism by which IRAK-4 kinase facilitates IL-1 β -induced signaling, recruitment of IRAK-1 or TRAF6 into the signaling complex was assessed. Our results indicate that IRAK-4 kinase activity is indispensable in IL-1R- and TLR-mediated signaling and expression of pro-inflammatory genes. To assess the role of IRAK-4 kinase activity in inflammatory responses *in vivo*, IRAK-4 KD mice are studied in animal models of chronic and acute inflammation.

01-45/P

NUCLEAR IMPORT AND PREPHOSPHORYLATION CONTROL INTERFERON-INDUCED STAT1 SERINE PHOSPHORYLATION

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The transcription factor Stat1 plays an essential role in responses to interferons and as such is indispensable for host defense to infections. Activation of Stat1 is achieved by phosphorylation at Tyr701 that is followed by nuclear translocation. For full transcriptional activity of Stat1 and its biological function, phosphorylation at Ser727 is also necessary. The molecular mechanisms underlying the interferon-induced Ser727 phosphorylation are poorly understood. Our data demonstrate that Stat1 nuclear translocation is required for interferon-gamma-induced Ser727 phosphorylation. In addition to nuclear import, interferon-induced Tyr701 phosphorylation is needed for Stat1 to become phosphorylated at Ser727 in both mouse and human cells. Thus, both interferon-induced events, tyrosine phosphorylation and nuclear translocation, are essential prerequisites for Stat1 to acquire phosphorylation at Ser727. Our data demonstrate that cellular localization and posttranscriptional modification are integral parts of the pathway that regulates Stat1 transcriptional activity. This two-level control mechanism, which may be used by other Stat proteins as well, represents a novel way of transcription factor regulation via targeting the transcription factor to the activating serine kinase and facilitating the recognition by the kinase.

01-46/P

IDENTIFICATION OF RESIDUES WITHIN VACCINIA VIRUS PROTEIN A52 THAT ARE CRITICAL FOR MAP KINASE ACTIVATION VIA TRAF6

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The Vaccinia virus (VV) genome encodes numerous proteins that modulate the host innate and adaptive immune responses, including proteins that disrupt Toll-like receptor (TLR) and cytokine function. We have previously shown that one of these proteins, A52, inhibits IL-1 and TLR-induced NF- κ B activation, NF- κ B gene induction and contributes to virulence. We subsequently showed that A52 also activates p38 MAP kinase, leading to enhanced TLR-dependent IL-10 induction. A52 mediates its effects on IL-1R/TLR signalling pathways by its interactions with components downstream of the receptor complexes, namely TRAF6 and IRAK2, two crucial signalling molecules of the IL-1/TLR pathways. Evidence so far suggests that A52-induced MAP kinase activation is primarily mediated by the TRAF6 interaction, and here we investigated the molecular mechanism of A52-TRAF6 binding and subsequent MAP kinase activation. Several proteins that interact with TRAF6 have been shown to contain a consensus sequence of amino acids (PXEXXAc/Ar), known as the TRAF6-binding motif. A search for this motif in A52 identified a putative motif at residues 149-154(RNEKLF), located within the C-terminal region of A52 that we previously showed to be required for interaction with TRAF6. Compellingly, mutation of a single residue within this motif (F154A) abrogated the ability of A52 to activate p38 and JNK MAP kinases but did not affect the ability of A52 to inhibit NF- κ B, suggesting that this residue is crucial for binding to or activating TRAF6. In contrast, the E151A mutant behaved the same as the wild-type protein. Surprisingly, the F154A mutant still bound to TRAF6. Here we have identified a single residue in A52 critical for MAP kinase activation. It is thus possible that the putative TRAF6-binding motif in A52 may be more important for activation of MAP kinases via TRAF6 rather than for TRAF6 binding.

01-47/O

CONTROL OF EIF2A KINASE PKR BY TYROSINE PHOSPHORYLATION: IMPLICATIONS IN GENE TRANSLATION, VIRUS REPLICATION AND CELL SIGNALING.

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Control of mRNA translation is one of the major regulatory events in eukaryotic gene expression. Recent research has established the existence of a protein kinase family in mammalian cells, whose members phosphorylate the alpha (α) subunit of the initiation factor eIF2 (eIF2 α) at serine 51 and regulate mRNA translation under various stress conditions. The interferon (IFN)-inducible double-stranded (ds) RNA-activated protein kinase PKR is the prototype of this family. Stress conditions activate PKR by autophosphorylation which leads to inhibition of host and viral protein synthesis and induction of apoptosis. PKR has been characterized as a serine/threonine kinase. Our studies demonstrate that PKR possesses tyrosine kinase activity and undergoes autophosphorylation at tyrosine (Tyr) residues 101, 162 and 293 *in vitro* and *in vivo*. Phosphorylation at these tyrosine residues enhances dsRNA binding-efficiency as well as the dimerization of PKR, which in turn favours the full-scale kinase activation and its substrate phosphorylation. Biologically, site-specific tyrosine phosphorylated PKR mediates the anti-viral and anti-proliferative activities of the eIF2 α kinase through its ability to inhibit protein synthesis. Furthermore, site-specific tyrosine phosphorylation of PKR is induced in response to IFNs through the activation of the receptor associated Janus kinases (Jaks). Induction of PKR tyrosine phosphorylation by IFNs represents a link between IFN signaling and translational control with implications in the regulation of IFN-mediated gene expression at the translational and transcriptional levels.

01-48/O

RECRUITMENT OF AN INTERFERON MOLECULAR SIGNALING COMPLEX TO THE MITOCHONDRIAL MEMBRANE

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Intracellular RNA virus infection is detected by the cytoplasmic DExD/H box RNA helicase RIG-I that plays an essential role in signaling to the host antiviral response. Recently, the adaptor molecule that links RIG-I sensing of incoming viral RNA to downstream signaling and gene activation events was characterized by four different groups; MAVS/IPS/VISA/Cardif contains an amino-terminal CARD domain and a carboxyl-terminal mitochondrial transmembrane sequence that localizes to the mitochondrial membrane. Furthermore, the Hepatitis C virus NS3/4A protease complex specifically targets MAVS/IPS/VISA/Cardif for cleavage as part of its immune evasion strategy. With a novel search program written in python, we also identified an uncharacterized protein, KIAA1271 (K1271), containing a single CARD-like domain at the N-terminus and a Leu-Val rich C-terminus that is identical to that of MAVS/IPS/VISA/Cardif. Using a combination of biochemical analysis, subcellular fractionation and confocal microscopy, we demonstrate that NS3/4A cleavage of MAVS/IPS/VISA/Cardif/K1271 results in its dissociation from the mitochondrial membrane and disrupts signaling to the antiviral immune response. Furthermore, VSV-induced expression of the non-canonical IKK-related kinase IKKε results in the co-localization of IKKε to the mitochondrial membrane in association with MAVS/K1271. Localization of both molecules was disrupted by NS3/4A expression, whereas mutation of the critical Cys508 to Ala was sufficient to maintain mitochondrial localization of MAVS/K1271 and IKKε in the presence of active NS3/4A. Constitutively expressed TBK1, on the other hand, localized with both the mitochondrial and the cytosolic fractions, in part in association with the early endosome; its localization was not disrupted by NS3-4A. These observations support a critical role for mitochondria in coordinating the innate antiviral response and provide evidence for the assembly of distinct IFN molecular signaling complexes that are ideally localized to respond to virus infection via activation of the IFN antiviral response.

01-49/P

PHYSICAL AND FUNCTIONAL MAP OF THE VIRUS-INDUCED TOLL-LIKE RECEPTOR SIGNALING PATHWAYS

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Signal transduction pathways are modular composites of interdependent sets of proteins that act in a coordinated fashion to transform environmental clues into a phenotypic response. Recent advances in protein purification and protein mass spectrometry have enabled the systematic analysis of signal transduction pathways by pathway proteomics. The pathway proteomics approach allows for the unbiased and comprehensive investigation of protein-protein-interactions in a given pathway, thereby enabling the construction of a coherent map of the pathway. It represents an ideal framework for integration of additional data from the literature and sets the ground for a more "systems biology" understanding of the process.

Viral infections continue to threaten human health. Although viruses are seemingly simple, antiviral therapies are by and large unavailable. Innate immunity to viral infection is triggered by conserved molecular patterns such as single- or double-stranded RNA. Those patterns are recognized by distinct Toll-like receptors (TLRs) that initiate the production of type-I interferons, thereby establishing an antiviral condition. Knowledge on the signaling pathways that mediate interferon production upon TLR stimulation is still patchy. Therefore, we are currently applying modern pathway proteomics technology to obtain a physical and functional map of the TLR signaling pathways that trigger interferon production upon viral infection.

01-50/P

IL-17A PROMOTES CANCER CELL SURVIVAL AND RESISTANCE TO APOPTOSIS VIA AKT-MEDIATED INACTIVATION OF CASPASES AND BAD

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IL-17A is produced mainly by memory activated CD4+ T cells. Because its receptor is ubiquitously expressed, IL-17 acts on many tissues. IL-17A regulates the immune system and fine-tunes hematopoiesis. IL-17A has been implicated in many human diseases including cancer, arthritis, lung diseases, and inflammation. Though most of these effects probably occur via induction of cytokine, I-CAM and PGE₂ production, the mechanisms of how IL-17A promotes cancer is not known. In this study we investigated whether IL-17A has the ability to promote resistance of leukemia cells to apoptosis. Apoptosis was induced in U937 leukemia cells with the HDAC inhibitor, sodium butyrate (NaB) in the absence or presence of IL-17A. NaB is a powerful inducer of apoptosis in cancer cells. Apoptosis versus survival was assessed by cell viability, caspases and Annexin V assays and Bcl-2 ELISA. We also examined the effects of IL-17A on leukemia cell growth. Our data shows that NaB (5 mM) causes greater than 60% cell death by apoptosis within 24 hr. Typically, NaB induces marked activation of caspases 3 and 9 as well as downregulation of Bcl-2. These apoptotic effects of NaB are significantly reversed by IL-17A. Clearly, IL-17A reduces the ability of NaB to cause cell death as well as downregulation of Bcl-2 by between 40 to 45%. Likewise, NaB-induced activation of caspases 3 and 9 were significantly reversed by IL-17A. We also observed that while the androgen-dependent (LNCaP) prostate cancer line barely expresses the IL-17A receptor, the androgen independent (C-42 and PC-4) prostate cancer cell lines markedly express the IL-17A receptor. Lastly, IL-17 stimulates Akt Ser 473 and Thr308 phosphorylation resulting in Akt activation and Akt-mediated phosphorylation and inactivation of GSK-3 and Bad. These results strongly suggest that IL-17A promotes cancer cell survival and resistance to apoptosis in a mechanism that involves Akt activation.

01-51/O

CRM1-DEPENDENT AND INDEPENDENT NUCLEAR EXPORT PATHWAYS REGULATE NUCLEOCYTOPLASMIC DISTRIBUTION OF STAT2

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Interferons (IFNs) reprogram gene expression through activation of Signal Transducer and Activator of Transcription (STAT) family members. Receptor-mediated tyrosine phosphorylation induces the dimerization of STAT proteins and their subsequent accumulation in the nucleus. In response to type I IFN heterodimers of STAT1 and STAT2 are formed. Since the movement between the cytoplasm and the nucleus is an important control mechanism for the activity of these STATs, we examined their dynamics of transport, using EGFP fusion proteins and photobleaching techniques. We found that STAT2 is permanently shuttling in unstimulated cells. A dominant nuclear export process that makes use of two different mechanisms is responsible for the observed cytoplasmic localization of the latent protein. One of these mechanism acts through the CRM1 nuclear export receptor, whereas the other is unknown. We further found that IFN type I treatment of the cells blocks the nuclear export of STAT2 while import is ongoing. We identified a region in the C-terminus of STAT2 that is responsible for CRM1-specific export. Nucleocytoplasmic shuttling of STAT2 is significantly different from that of STAT1. STAT1 is also shuttling in the absence of IFN, but the exchange rate in unstimulated cells is more than ten times lower than that of STAT2. Using bimolecular fluorescence complementation we detected STAT1-STAT2 heterodimer formation in the absence of IFN and analysed the rate of nucleocytoplasmic shuttling for defined combinations of mutant STAT proteins. From these we developed a model for IFN type I induction in which the receptor-mediated heterodimerization overcomes the slow nuclear import of STAT1 and blocks the strong STAT2 export activity leading to the accumulation of both signal transducers in the nucleus.

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EVIDENCE FOR STAT1 ARGININE METHYLATION

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Arginine methylation of STAT1 has been described as an important post-translational modification that regulates type I interferon (IFN) induced transcription (Cell, 2001, 104:731). Using the dimethylarginine (DMA) immunoprecipitation method described in this publication, we have found a decreased methylation of STAT1 in cells expressing hepatitis C virus (HCV) proteins, in liver extracts from HCV transgenic mice, and in liver biopsies from patients with chronic hepatitis C (Gastroenterology, 2004, 126:263). However, there have been two publications challenging the concept of STAT1 methylation (Cell, 2004, 119:587; J Biol Chem, 2005, 280:21700). We therefore set out to find further evidence for or against STAT1 methylation. We first used purified full length and N-terminal STAT1-GST fusion proteins, purified protein arginine methyltransferase 1 (PRMT1) and C¹⁴-AdoMet in an *in vitro* methylation assay, but failed to detect incorporation of C¹⁴-labeled methyl groups into STAT1. However, the purified STAT1-GST fusion proteins could be detected with DMA antibodies even before submitting them to the *in vitro* methylation assay. It is possible that C¹⁴ incorporation in the *in vitro* assay failed because the STAT1 fusion proteins were already fully dimethylated on arginine. We therefore tested if C¹⁴-labeled methyl groups would be incorporated into newly synthesized STAT1 *in vivo*. Indeed, incubation of Huh7 cells with C¹⁴-AdoMet for 4 days resulted in an increasing labeling of STAT1 to a maximum that is reached after 48 hours. Further culture of the cells in medium without C¹⁴-AdoMet showed a decline of the STAT1 specific C¹⁴ signal with a half-life of 20 hours. We then tested and confirmed the specificity of the DMA immunoprecipitation method for detection of methylated STAT1 using STAT1 deficient mouse embryonic fibroblasts (MEFs). Specific methylation of STAT1 was further confirmed by transfecting a flag-tagged STAT1 into MEFs and *in vivo* labeling with C¹⁴-AdoMet. Finally, preliminary mass spectrometry (MS) analysis revealed methylated STAT1 peptides.

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STAT3 IN THE MITOCHONDRIA REGULATES THE ACTIVITY OF SELECTIVE COMPLEXES OF THE ELECTRON TRANSPORT CHAIN

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GRIM-19, which is responsible for interferon beta (IFN β) induced apoptosis of breast cancer cells, has been shown to bind to Stat3 and inhibits its ability to function as a transcriptional activator. GRIM-19 is an essential component of complex I of the mitochondrial electron transport chain (ETC). We present evidence that Stat3 is present in mitochondria isolated from a variety of cultured cells as well as mouse tissues. Compared with wild type cells, IL-7-dependent pro B cells that do not express Stat3 show decreased concentrations of cellular ATP, and about a 75% decrease in the activities of complex I and

V of the ETC. Reconstitution of Stat3^{-/-} cells with Stat3 α restores mitochondrial function. Stat3^{-/-} cells reconstituted with Stat3 α that has either a mutation in its tyrosine phosphorylation site (Y705) or its DNA binding domain also restores function, as well as Stat3 α which has been targeted to the mitochondria with a mitochondrial localization sequence. Mutation of serine 727, which has been reported to be required for binding of Stat3 to GRIM-19, does not reconstitute mitochondrial function in Stat3-null cells. Since expression of Stat3 regulates the activity of complexes I and V of the ETC, we examined whether this transcription factor is associated with the mitochondrial ETC. Mitochondria were isolated from mouse heart and liver. The ETC was solubilized and the 5 complexes were isolated. Immunoblots revealed that Stat3 is a component of complex I and V. Interestingly when mice were injected with LPS, (which induces serine phosphorylation of Stat3), the association of Stat3 with complexes of the ETC is altered. These results indicate a novel role for Stat3 in regulation of the mitochondrial ETC. Since inflammation and ischemia have been reported to depress the ETC, we hypothesize that Stat3 plays an integral role in controlling changes in respiration under conditions of cellular stress.

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PROTEOMIC AND NETWORK ANALYSIS OF ISG15-CONJUGATED PROTEINS IN THE MACROPHAGE

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The ubiquitin-like molecule ISG15 is one of the most highly upregulated proteins following type I interferon or lipopolysaccharide (LPS) stimulation, indicating a probable role in the innate immune response. Like ubiquitin, ISG15 is conjugated to a large number of intracellular protein targets. Interestingly, influenza B virus has developed a mechanism to inhibit ISGylation, indicating an evolutionarily conserved role of ISG15 in viral immunity. However, ISG15 knockout mice have not yet revealed a biological role for ISG15. In an effort to understand its role during the innate immune response, we have identified approximately 200 putative ISG15 targets in the LPS-stimulated RAW 264.7 murine macrophage cell line by proteomic analysis. This analysis utilized a newly designed rapid tandem affinity purification tag (RAPID-tag) combined with SDS-PAGE, reverse phase microcapillary liquid chromatography, tandem mass spectrometry, automated sequence database searching through Sequest, and the powerful statistical analysis algorithms PeptideProphet and ProteinProphet to identify with high confidence the ISG15 interactome. To date 19 out of 20 putative targets tested in a transfection-based ISGylation assay have been confirmed to be ISGylated. Using network visualization software, analysis of ISG15 targets with the highest ProteinProphet confidence scores predicts several biological functions where ISG15 may play a role, such as proliferation, focal contact, and phagocytosis. Ultimately, this information will allow us to analyze ISGylated targets inside a network model of Toll-like receptor signaling in the macrophage, giving us insight not only into the biological effects of ISG15, but, also, into the complex system of macrophage signaling employed to ward off and fight infection.

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INTRACELLULAR SIGNALING PATHWAYS FOR IFN-DEPENDENT ANTIVIRAL RESPONSES

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Viral infection leads to IFN production through at least two distinct signaling systems. Engagement of some Toll-like receptors (TLRs) can lead to IFN production following transmembrane signaling events, while cytoplasmic recognition of viral infection can lead to IFN production through a separate signaling pathway. In most cells and tissues, both signaling pathways converge on the same effector proteins, which lead to the phosphorylation of IRF3 and IRF7 by the kinases TBK1 and IKK- ϵ . It has become increasingly clear that intracellular

topology plays an important role in these pathways. For instance, a striking difference between the signaling capabilities of plasmacytoid and myeloid dendritic cells appears to be controlled by the intracellular organelles involved in viral recognition and signal propagation. Similarly, different viruses replicate at distinct locations within the cell, and yet still activate the same end result of IFN production.

We have examined the intracellular signaling topology of IFN induction by comparing the cellular response to the model negative strand RNA and DNA viruses, NDV and HSV1. In this process, we have examined the requirements for the intracellular signaling components Myd88, TRIF, Unc-93b1, RIG-I, MAVS/IPS-1, TBK1, IKK- ϵ , IRF3, and IRF7. Remarkably, while NDV requires the activity of the RIG-MAVS-TBK-IRF axis for IFN production, these components are at least partially redundant for antiviral signaling in response to HSV infection, which remains effective in the absence of these signaling molecules. Moreover, viral virulence factors that block antiviral responses, such as influenza virus NS1 and poxvirus E3L proteins are capable of inhibiting IFN production by the RIG-MAVS-TBK-IRF axis, they are unable to facilitate the evasion of host innate immunity to HSV. In spite of their role in viral virulence through IFN inhibition, the mechanisms underlying the inhibitory action of E3L and NS1 remain unclear. To elucidate their mode of action, we have investigated protein-protein interactions during the inhibition of signaling by E3L. To this end, we have performed yeast two-hybrid assays and fluorescence resonance energy transfer experiments between E3L and various signaling components, as well as between different members of the signaling cascade. Moreover, we have evaluated the intracellular locations of these cytoplasmic signaling components in both resting cells and in cells responding to viral infections. Unexpectedly, the intracellular locations of the components of these essential signaling cascades do not fit a straight forward model of a cytoplasmic signaling cascade. Instead, our results reveal a complex involvement of both cytoplasmic and organelle subcellular locations during antiviral signaling.

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HEAT SHOCK PROTEIN 27 IS REQUIRED FOR STABILISATION OF INTERLEUKIN-1-INDUCED CYCLO-OXYGENASE-2 AND INTERLEUKIN-6 MRNAS

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Heat shock protein 27 (HSP27) is a major protein that is phosphorylated in response to interleukin (IL)-1 treatment of fibroblasts. It is

phosphorylated by MK2, the kinase downstream of p38 mitogen-activated protein kinase (MAPK). Despite these observations, the role of HSP27 in inflammation has remained unclear. To examine its role in inflammatory gene expression the protein was depleted in HeLa cells by RNA interference. Suppression of HSP27 expression by short interfering RNAs inhibited IL-1-induced cyclooxygenase-2 (COX-2) and IL-6 protein and mRNA. IL-1-induced IL-8 protein expression was also inhibited upon HSP27 suppression. The induction of COX-2 and IL-6 protein by phorbol ester, a stimulus that does not activate the p38 MAPK pathway, was unaffected by HSP27 depletion. The inhibition of IL-1-induced COX-2 expression upon HSP27 depletion was caused by reduced stability of these mRNAs. The effect was specific to only certain mRNAs as the stability of Bcl-x, GADD-45, c-Fos, p21, Bax, Mcl-1 and L32 mRNAs was unaffected.

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STABILISATION OF TNF, IL-1 α AND IL-6 MRNAS BY P38 MITOGEN-ACTIVATED PROTEIN KINASE IN MURINE MACROPHAGES REQUIRES TRISTETRAPROLIN

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The p38 mitogen-activated protein kinase (MAPK) signalling pathway plays an important role in the regulation of inflammatory gene expression. p38 MAPK stabilises inflammatory mRNAs via AU-rich elements (ARE) present in the 3' untranslated region of these mRNAs. The mRNA destabilising protein, tristetraprolin (TTP) is known to bind the ARE of tumour necrosis factor (TNF) mRNA and promote its deadenylation and decay. We show that in lipopolysaccharide-treated RAW 264.7 cells, p38 MAPK stabilises endogenous TNF mRNA by inhibiting its deadenylation. In TTP-null macrophages the destabilisation of lipopolysaccharide-induced TNF mRNA following p38 MAPK blockade is reduced but not ablated. This suggests that multiple ARE-binding proteins, one of which is TTP, may be required for the decay of TNF mRNA. We find that TTP is also required for the destabilisation of IL-6 and IL-1 α mRNA upon p38 MAPK inhibition. The data are consistent with a model in which the p38 MAPK pathway stabilises inflammatory mRNAs by inhibiting the mRNA destabilising function of TTP.